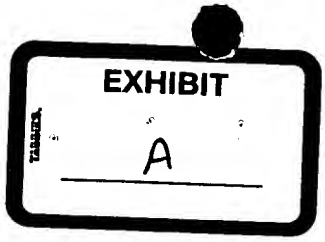

ANNEX

The aim of this study was to investigate the properties of $VEGF_{114}$ - a novel VEGF isoform. In order to characterized $VEGF_{114}$ it was over-expressed as a protein in endothelial cells (EC) and smooth muscle cells (SMC) using adeno- and retroviral expression systems and its biological properties were compared with those of the known $VEGF_{165}$ isoform.

Methods

I. Generation of recombinant viral vectors:

Generation of recombinant Adenoviral vectors encoding *$VEGF_{114}$* gene

The recombinant adenoviral vector expressing the human $VEGF_{114}$ gene was constructed by a modified AdEasy protocol (Vogelstein B. PNAS 1998). A 426bp Bam HI-Xho I fragment of $VEGF_{114}$ cDNA was inserted into the Bgl II- Xho I site in the pAdShuttle-CMV vector under the control of the CMV promoter. The shuttle vectors were linearized by PmeI digestion and purified by Qiaquick gel extraction kit. The linearized shuttle vector and pAdEasy-1 were co-transformed by electroporation into competent BJ5183 cells. Positive clones containing the recombinant adenoviral vectors were selected according to PCR and restriction map analysis. The recombinant adenoviral plasmids were linearized by PacI digestion, purified and transfected into 293 cells using Lipofectamine 2000 (Gibco BRL, USA). Seven days after transfection CPE occurred and 100% of the cells expressed GFP. The cells were harvested and viral extracts were further amplified in 293 cells. The viral stock titer was determined by serial dilution assay in 293 cells and ranged ~10^{11} pfu/ml. The expression of the transgene was confirmed by western analysis of the infected cells condition media.

Construction of retroviral vectors for expression of *$VEGF_{114}$* or co-expression of *$VEGF_{114}$* and GFP

Recombinant retroviral vector LXSN-VEGF encoding the human VEGF gene was constructed by inserting the human $VEGF_{114}$ cDNA 426bp EcoR1-Xho1 fragment into the EcoR1-Xho1 site of plasmid pLXSN (# K1060-B Clontech, USA) under the control of Mo-MULV 5' long terminal repeat (LTR).

The recombinant retroviral vectors expressing the human $VEGF_{114}$ and /or the EGFP genes were constructed by cloning into pLXSN plasmid in two steps. First, an IRES- EGFP EcoRI-HpaI fragment (1400 bp) excised from pIRES2-EGFP (#6029-1 Clontech) was inserted into EcoRI-HpaI sites in pLXSN for construction of the control plasmid pLXSN-IRES-EGFP. The second step was construction of pLXSN-VEGF114-IRES-EGFP by cloning of human VEGF114 EcoRI-Xho I fragment (426 bp) into EcoRI-Sal I site in pLXSN-IRES-EGFP. The expression cassettes are regulated by the Mo-MULV 5' long terminal repeat (LTR).

Generation of pseudotyped recombinant retroviral vectors encoding *$VEGF_{114}$*

For retroviral vector production, pLXSN- $VEGF_{114}$-IRES-GFP vector or pLXSN- $VEGF_{114}$ was transfected into 293FLYA packaging cells using Lipofectamine (Gibco BRL, USA). After 48 hours, supernatant from confluent

cultures of viral producer cells was collected, filtered (0.45 μm) and added to 293 FLYGALV packaging cells. Transduced cells were grown under G418 selection (400 μg/ml) and individual colonies were collected and screened for EGFP expression, using an inverted fluorescent microscope, and $VEGF_{114}$ expression by Western analysis of transduced cell-conditioned medium. The viral titer of each colony was determined via transduction of TE671 cells and the titers of ~10^6 ffu/ml were obtained. The colonies with the highest-titers were selected and supernatant was collected freshly for transduction of EC and SMC.

II. Verifying transgene expression after gene transfer

Cell culture

Endothelial cells (EC) were isolated from human saphenous veins (HSVEC), and cultured on gelatin-coated dishes in M20 containing M-199 Medium (Biological Industries, Israel) supplemented with 20% FCS, 2mM L-Glutamin, 100 units/ml penicillin, and 0.1 mg/ml streptomycin, 100μg/ml Heparin (Sigma) and 2ng/ml bFGF (Enco). Human EC were identified by immunohistochemistry analysis with anti Von-Willebrand factor specific antibodies (Zymed, USA). Smooth muscle cells (SMC) were cultured by explant outgrowth from human saphenous veins (HSVSMC) and left internal mammary arteries (HLSMC). Cells were cultured in Dulbeco's Modified Eagles Medium (DMEM) (Biological Industries, Israel) supplemented with 10% human serum, 2 mM L-Glutamin, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 2ng/ml bFGF. SMC were identified by immunohistochemistry analysis using specific anti-α smooth muscle actin antibodies (Zymed, USA).

The packaging cell lines 293-FLYA, 293-FLYGALV and TEFLYGA (obtained from Dr F.L. Cosset -Lion, France) were grown in DMEM supplemented with 10% FCS, 2mM L-Glutamin, 100 units/ml penicillin, 0.1mg/ml streptomycin, 6μg/ml blasticidin and 6μg/ml phleomicin.

Infection of EC and SMC with recombinant adenovirus vectors

Endothelial cells and smooth muscle cells were infected with recombinant adenoviral vectors as follows: the cells were seeded at 70% confluence on fibronectin pre-coated plates (4.5μg/ml) 20 hours prior to infection and grown in complete medium (M20). At the day of infection the medium was replaced with fresh M199 (without serum) and the recombinant virus was added at Multiplicity of Infection of 1000 (MOI=1000), which mean 1000 viral particles per cell. The cells were incubated for 90 minutes with gentle tilt every 20 minutes. After infection the virus-containing medium was replaced with complete medium (M20).

Transduction of EC and SMC with recombinant retroviral vectors

Transduction of endothelial cells and smooth muscle cells with the retroviral vectors was performed as followed: EC (passage 4-9) were seeded (10^5 cells per 35-mm well) in fibronectin-coated plates (4.5μg/ml) and grown in complete medium for 24 hours. One hour prior to transduction, the medium was replaced with serum free M199 containing 0.1mg/ml of the cationic polymer DEAE-dextran (Sigma). After pre-conditioning, the cells were washed three times with phosphate-buffered saline (PBS). Transduction was performed by incubation of the cells for 4 hours, with supernatants containing viruses collected and filtered (0.45μ) freshly from the virus

producing packaging cell lines. At the end of the incubation the medium was replaced with fresh M20 medium.

$VEGF_{114}$ over-expression by infected EC and SMC

Western blot analysis

$VEGF_{114}$ protein expression by adenoviral or retroviral infected EC and SMC was detected by ELISA or western blot analysis of the conditioned medium. 24 hours post infection the medium was changed to serum free medium and cells were grown for additional 48 hours.

For western blot analysis the samples of the conditioned medium (30µl) were separated on 8% SDS polyacrylamide gel under reducing conditions, and electrotransferred to nitrocellulose membrane (Shleicher & Schull). The blots were blocked with 0.1% skim milk in TBS containing 0.3% tween-20 (TBST) for 1 hour at room temperature using gentle agitation. The blots were incubated with primary antibody diluted in blocking solution for 2 hours at room temperature. 1:500 dilution of polyclonal rabbit anti-VEGF antibody (#SC 152 Santa- Cruz, USA) was used for *$VEGF_{114}$* detection. Following the incubation the blots were washed three times with TBST and incubated for 1 hour at room temperature with anti rabbit peroxidase-conjugate antibody (Sigma) diluted 1:7000 in TBST. After three washes with TBST bound antibody was visualized using the ECL reagents (Sigma) and exposed to X-ray film.

To detect $VEGF_{114}$ protein concentration in the conditioned medium of viral infected cells, Human VEGF ELISA kit (Oncogene, Cat# QIA51) was used according to the manufacture instructions.

III. Physiological Effects of *$VEGF_{114}$* gene transfer

Proliferation assay with conditioned medium of rAd *$VEGF_{114}$* infected EC

Conditioned medium containing $VEGF_{114}$ was collected by following way: EC (passages 5-11) were seeded in 6-well plate at the concentration 150.000 cells/well and infected with rAd $VEGF_{114}$, rAd $VEGF_{165}$–GFP or rAdGFP. As additional control group served non-infected cells. 24 hrs after infection the medium was changed to serum free medium, the cells were grown for additional 48 hours. The conditioned medium was collected and used for the following proliferation assay.

To perform proliferation assay EC were seeded at the concentration $2x10^4$ cells/ well in 24 wells plate pre-coated with fibronectin (4.5µg/ml) in M199 containing 5% FCS. The assay was performed in triplicates. Increasing volumes of conditioned medium containing $VEGF_{114}$, VEGF or control were added each other day. Proliferation rate was detected 6-7 days after infection by cell counting using cell coulter.

Proliferation assay with recombinant retro *$VEGF_{114}$* transduced EC

EC (passages 5-11) were tranduced with recombinant retro $VEGF_{114}$–GFP, $VEGF_{165}$–GFP or GFP viruses. As additional control group served non-infected cells. Retrovirus transduced cells were isolated by selection with 250 µg/ml of G418. After selection the cells were seeded at the concentration $6x10^5$ cells/well in 6 wells plate pre-coated with fibronectin (4.5µg/ml) in M199 containing 20% FCS. The assay was

producing packaging cell lines. At the end of the incubation the medium was replaced with fresh M20 medium.

$VEGF_{114}$ over-expression by infected EC and SMC

Western blot analysis

$VEGF_{114}$ protein expression by adenoviral or retroviral infected EC and SMC was detected by ELISA or western blot analysis of the conditioned medium. 24 hours post infection the medium was changed to serum free medium and cells were grown for additional 48 hours.

For western blot analysis the samples of the conditioned medium (30µl) were separated on 8% SDS polyacrylamide gel under reducing conditions, and electrotransferred to nitrocellulose membrane (Shleicher & Schull). The blots were blocked with 0.1% skim milk in TBS containing 0.3% tween-20 (TBST) for 1 hour at room temperature using gentle agitation. The blots were incubated with primary antibody diluted in blocking solution for 2 hours at room temperature. 1:500 dilution of polyclonal rabbit anti-VEGF antibody (#SC 152 Santa- Cruz, USA) was used for *$VEGF_{114}$* detection. Following the incubation the blots were washed three times with TBST and incubated for 1 hour at room temperature with anti rabbit peroxidase-conjugate antibody (Sigma) diluted 1:7000 in TBST. After three washes with TBST bound antibody was visualized using the ECL reagents (Sigma) and exposed to X-ray film.

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Proliferation assay with recombinant retro *$VEGF_{114}$* transduced EC

EC (passages 5-11) were tranduced with recombinant retro $VEGF_{114}$–GFP, $VEGF_{165}$–GFP or GFP viruses. As additional control group served non-infected cells. Retrovirus transduced cells were isolated by selection with 250 µg/ml of G418. After selection the cells were seeded at the concentration $6x10^5$ cells/well in 6 wells plate pre-coated with fibronectin (4.5µg/ml) in M199 containing 20% FCS. The assay was

performed in duplicates. Proliferation rate was detected 7 and 10 days after seeding by cell counting using cell coulter.

In-vitro angiogenesis assay – sprouting assay in collagen

In-vitro angiogenesis was examined using endothelial cell (EC), smooth muscle cell (SMC), and mixed EC and SMC coculture sprouting from spheroids in collagen three-dimension matrix. The generation of spheroids was performed as described by Korff T. and Augustin H. (JBC 1998). EC were tagged with DiI-291 red fluorescent marker prior to mixing. 750 cells (EC or SMC) per single culture spheroid or 375 cells from each type (EC and SMC) for coculture spheroid were suspended in culture medium containing 0.25% (w/v) carboxymethylcellulose, seeded in nonadherent round-bottom 96-well plates (Nunc, Denemark). During 24 h incubation at 37°C, 5% CO_2 the suspended cells form a single spheroid per well of defined size and cell number. The spheroids generated than embedded in collagen gels. A collagen stock solution was prepared prior to use by mixing 8 vol acidic collagen extract of rate tails (equilibrated to 2 mg/ml, 4°C) with 1 vol 10XM199 (Gibco BRL, USA); 1 vol neutralization solution containing 0.34 N NaOH and 7.5% $NaHCO_3$ to adjust the pH to 7.4. This stock solution (0.5 ml) was mixed with 0.5 ml room temperature medium M199 with 40% human serum containing 0.5% (w/v) carboxymethylcellulose to prevent sedimentation of spheroids before polymerization of the collagen gel. The spheroids (20-30) containing gel was rapidely transferred into prewarmed 24-well plates and allowed to polymerize. The gels were incubated at 37°C, 5% CO_2 and documented by digital video camera (DXM1200 Nikon, Japan).

Apoptosis assay

Retroviral vector transduced HSVEC were seeded onto gelatinized 6-well plates ($2*10^5$ cells/well) in M199 medium supplemented with 20% (v/v) fetal calf serum (FCS) and incubated for 24 h.

In order to induce apoptosis, the cells were "pulse" exposed to H_2O_2: the medium was changed to 1ml of modified-PBS + 150μM H_2O_2 for 1 hour at 37^0 and then changed to M199 medium supplied with 2% FCS and incubated for 24 h.

All floating cells and all adherent cells were collected to the same tube after trypsinization (0.05% trypsin-EDTA). The cells were then pelleted, washed with ice-cold PBS, and resuspended in 1x binding buffer at a concentration of $1*10^6$ cells/ml. Annexin V-PE and 7-AAD (ready-made solutions) were added to the cells, mixed and incubated for 15 minutes at RT in the dark. Finally, 1x binding buffer was added to a final volume of 0.5ml. Analysis by a FACScan flow cytometer equipped with a doublet discriminating module was performed within 1 hour.

For each checked cell we used two controls. One of unstained cells and the other of Annexin V stained alone, in order to reduce the GFP signal that interferes with the PE signal.

Results

Sequence analysis of $VEGF_{114}$ cDNA

Both strands of the $VEGF_{114}$ cDNA were sequenced and the nucleotide sequence was compared with published VEGF sequence. $VEGF_{114}$ cDNA consists of exons 1-5 which contain information required for the recognition of the known VEGF receptors VEGFR1 and VEGFR2 and are present in all VEGF isoforms (Keyt, B.A.,

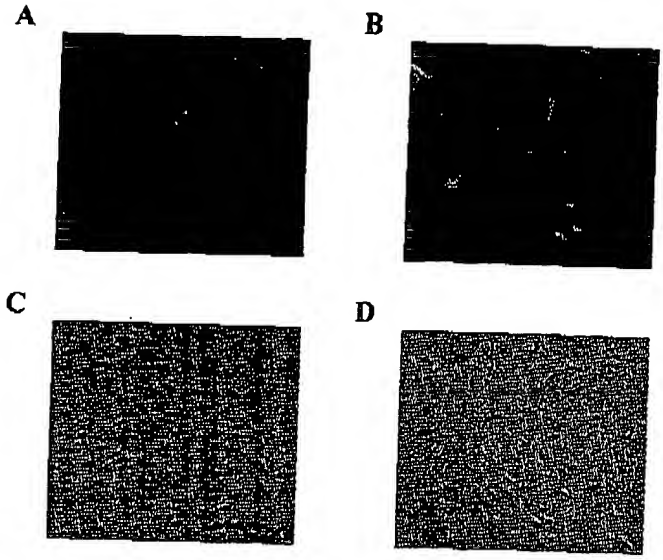


Figure 1: Transduction of EC and SMC with retro $VEGF_{114}$-GFP. HSVEC (A and C) and HSVSMC (B and D) were transducted with retro $VEGF_{114}$-GFP and visualized by either fluorescent inverted microscope (A and B) or by light inverted microscope (C and D).

1997). The $VEGF_{114}$ cDNA lacks exons 6 and 7 encoding heparin binding domains and presenting in $VEGF_{145}$ and $VEGF_{165}$ respectively. $VEGF_{189}$ contains both of the heparin binding domains. The $VEGF_{114}$ cDNA also lacks exon 8 which exists in all VEGF isoforms. To conclude, $VEGF_{114}$ cDNA resembles structure of $VEGF_{121}$ cDNA without exon 8. We have not found any mutation in the $VEGF_{114}$ cDNA.

$VEGF_{114}$ expression in EC and SMC

To study the properties of $VEGF_{114}$, $VEGF_{114}$ cDNA was expressed in EC and SMC using adeno- and retroviral expression systems. Transgene expression was verified by detection of green fluorescence. Transduction of EC and SMC by retroviral vector encoding $VEGF_{114}$-GFP demonstrated the production of the GFP protein (figure 1).

$VEGF_{114}$ protein expression was examined by western blot analysis (~18 kD protein corresponding to the $VEGF_{114}$ protein) after infection of EC with adenoviral vector encoding $VEGF_{114}$ (figure 2) and retroviral vector (figure 3).

To detect $VEGF_{114}$ protein concentration in the conditioned medium of adenovirus infected cells we used Human VEGF ELISA kit (Oncogene, Cat# QIA51). The concentrations of $VEGF_{114}$ and $VEGF_{165}$ proteins were 2.7ng/μl and 0.3ng/μl respectively.

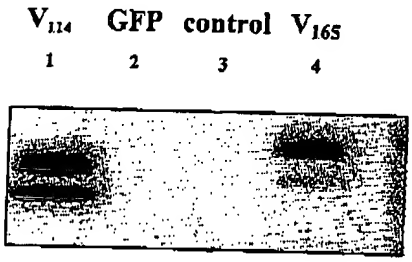


Figure 2: Western blot analysis of $VEGF_{114}$ expression by rAd infected EC.
EC were infected by adenoviral vectors as previously described. 24 hours post infection the medium was changed to serum free medium and cells were grown for additional 48 hours. Samples of the conditioned medium (30µl) were loaded on 12% SDS polyacrylamide gel, transferred to nitrocellulose membrane and the blots were incubated with anti-VEGF antibody. Following exposure to a peroxidase-conjugate secondary antibody the blots were developed with ECL reagents and exposed to X-ray film.

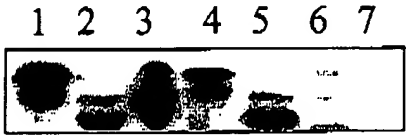


Figure 3: Western blot analysis of $VEGF_{114}$ expression by retro transduced EC and SMC.
EC and SMC were transduced by retro vectors as previously described. 24 hours post infection the medium was changed to serum free medium and cells were grown for additional 48 hours. Samples of the conditioned medium (30µl) were loaded on 12% SDS polyacrylamide gel, transferred to nitrocellulose membrane and the blots were incubated with anti-VEGF antibody. Following exposure to a peroxidase-conjugate secondary antibody the blots were developed with ECL reagents and exposed to X-ray film. Lanes are as follows: 1. Control non-transducted SMC; 2. retro $VEGF_{114}$ transducted SMC; 3. retro $VEGF_{165}$ transducted SMC; 4. positive control of $VEGF_{165}$; 5. retro $VEGF_{114}$ transducted EC; 3. retro $VEGF_{165}$ transducted EC; 7. Control non-transducted EC

$VEGF_{114}$ biological activity

The biological activity of the recombinant $VEGF_{114}$ was tested using proliferation assay and in-vitro angiogenesis assay and apoptosis assay.

The proliferation assays were performed with 1) naive EC by addition of conditioned medium from rAd $VEGF_{114}$ infected EC or 2) with recombinant retro $VEGF_{114}$ transduced EC. In both kinds of experiments $VEGF_{114}$ induces proliferation of EC, but it was less potent than $VEGF_{165}$ (figures 4 and 5).

To study the effects of $VEGF_{114}$ expression was tested on EC biology EC sprouting from spheroids in collagen three-dimension matrix. Endothelial cells over expressing $VEGF_{114}$ after retroviral gene transfer had no effect on EC sprouting while EC over expressing $VEGF_{165}$ exhibited an increased sprouting (figure 6A). The same results were obtained from co-culture experiments with retrovirus GFP transduced EC and $VEGF_{165}$/GFP or $VEGF_{114}$/GFP retro transduced SMC (figure 6B).

To examine whether over-expression of $VEGF_{114}$ or $VEGF_{165}$ in EC protect the cells from undergoing apoptosis Annexin V-PE staining which can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation was used. FACScan flow cytometer analysis was preformed to detect Annexin V-PE staining. As shown in figure 7 $VEGF_{165}$ protected EC from apoptosis much better than $VEGF_{114}$.

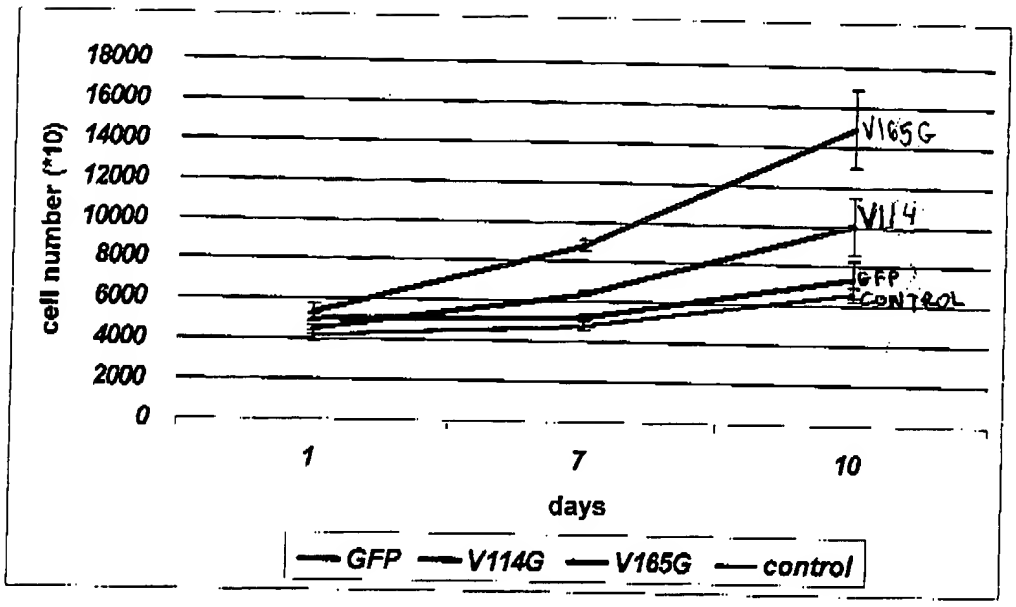
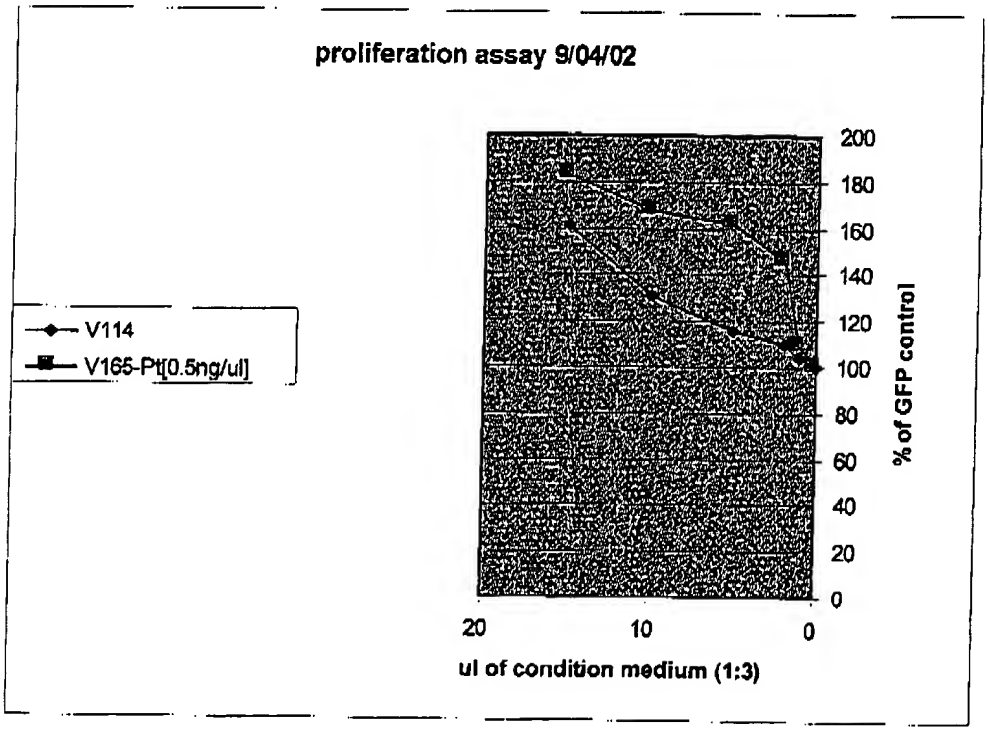
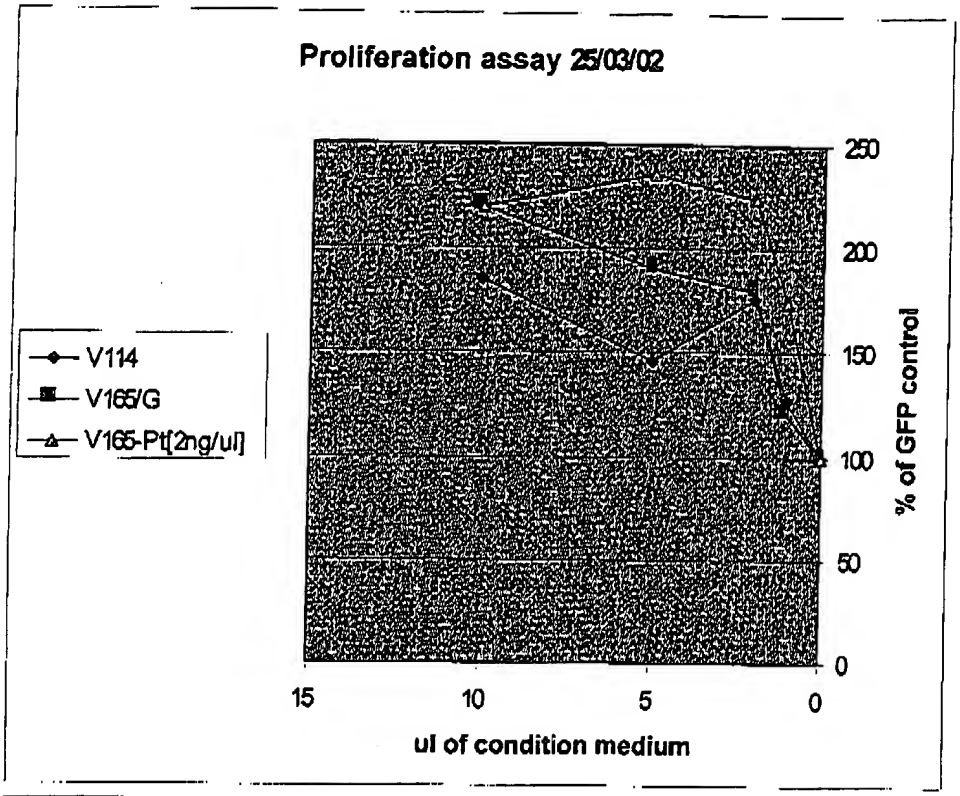


Figure 4: Proliferation assay with recombinant retro $VEGF_{114}$ transduced EC
EC were tranduced with recombinant retro $VEGF_{114}$-GFP, $VEGF_{165}$-GFP or GFP viruses. As additional control group served non-infected cells. The assay was performed as described in methods. Proliferation rate was detected 7 and 10 days after seeding by cell counting using cell coulter.

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5
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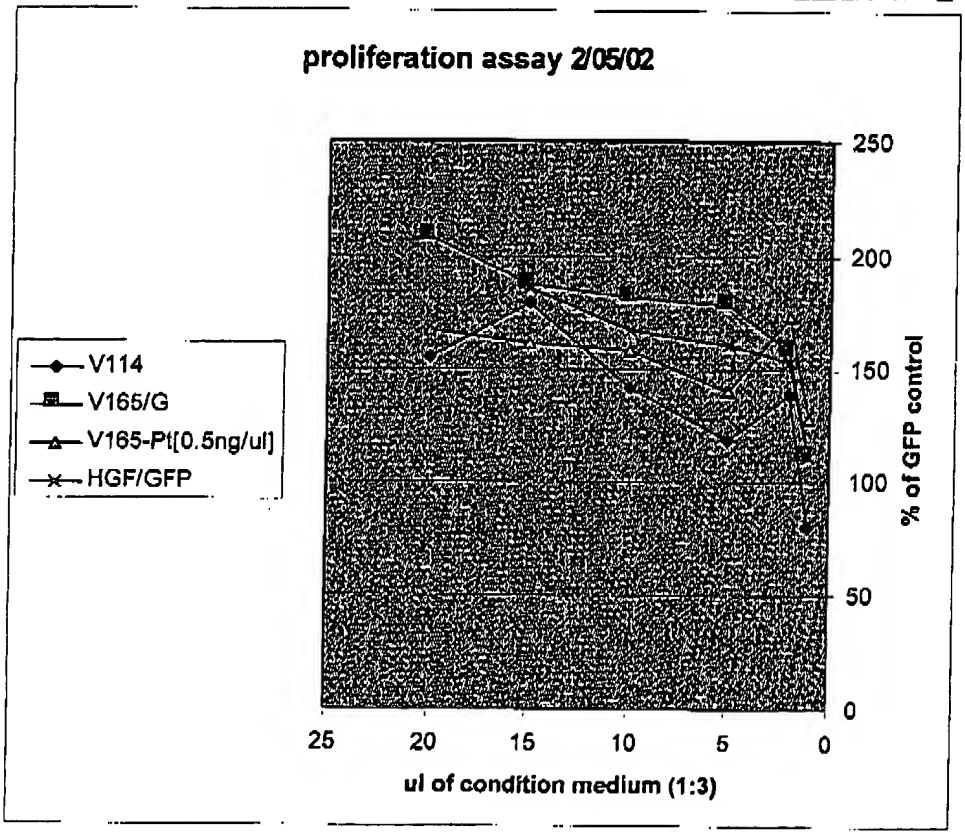
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15
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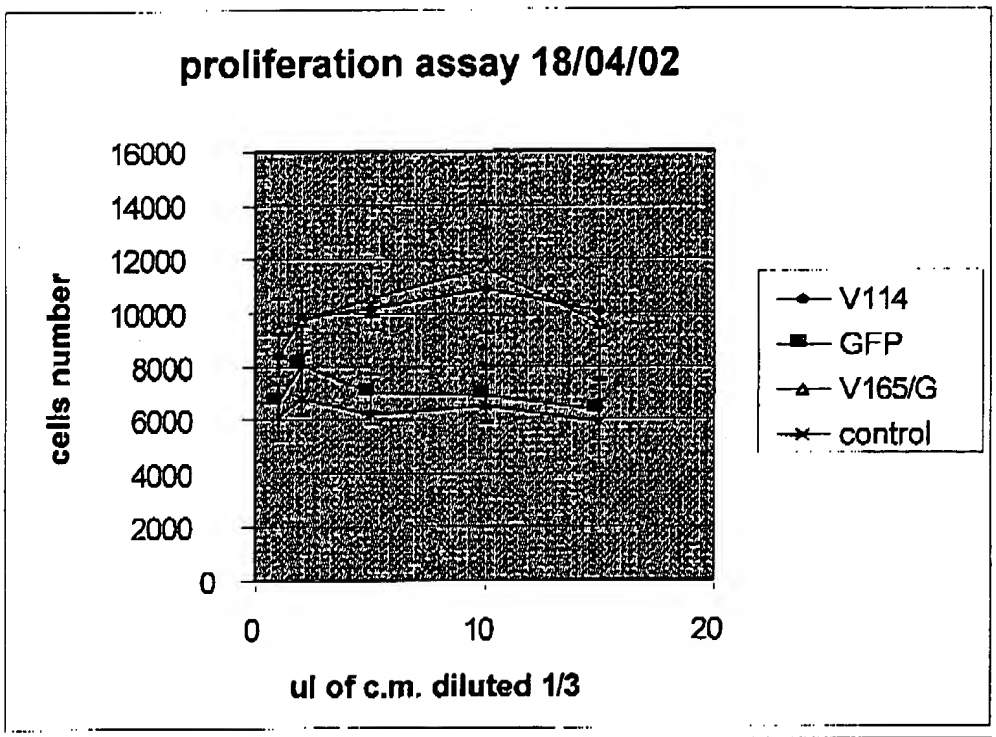
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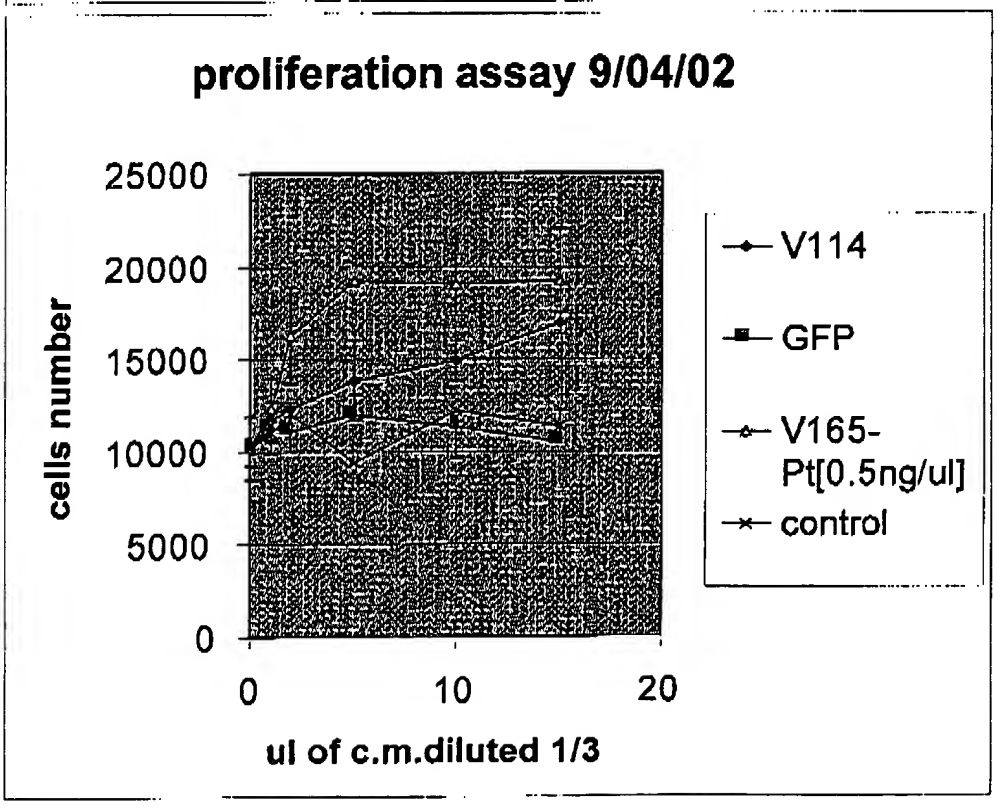
control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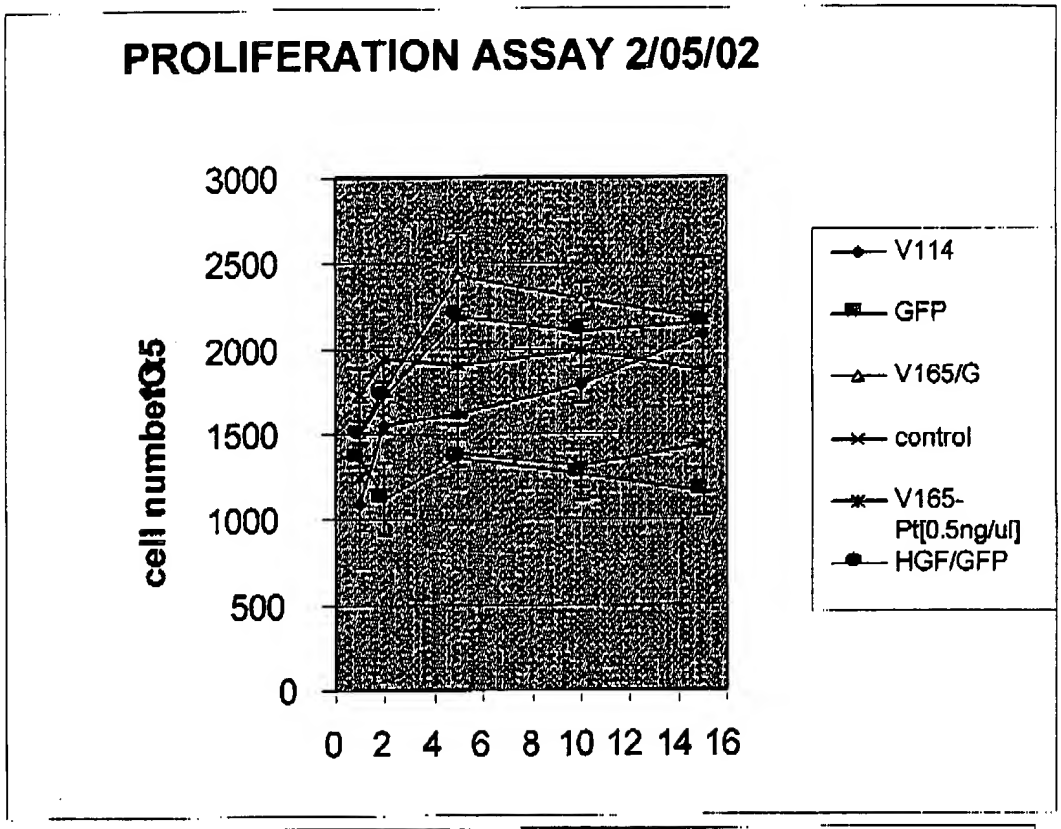
PROLIFERATION ASSAY 2/05/02
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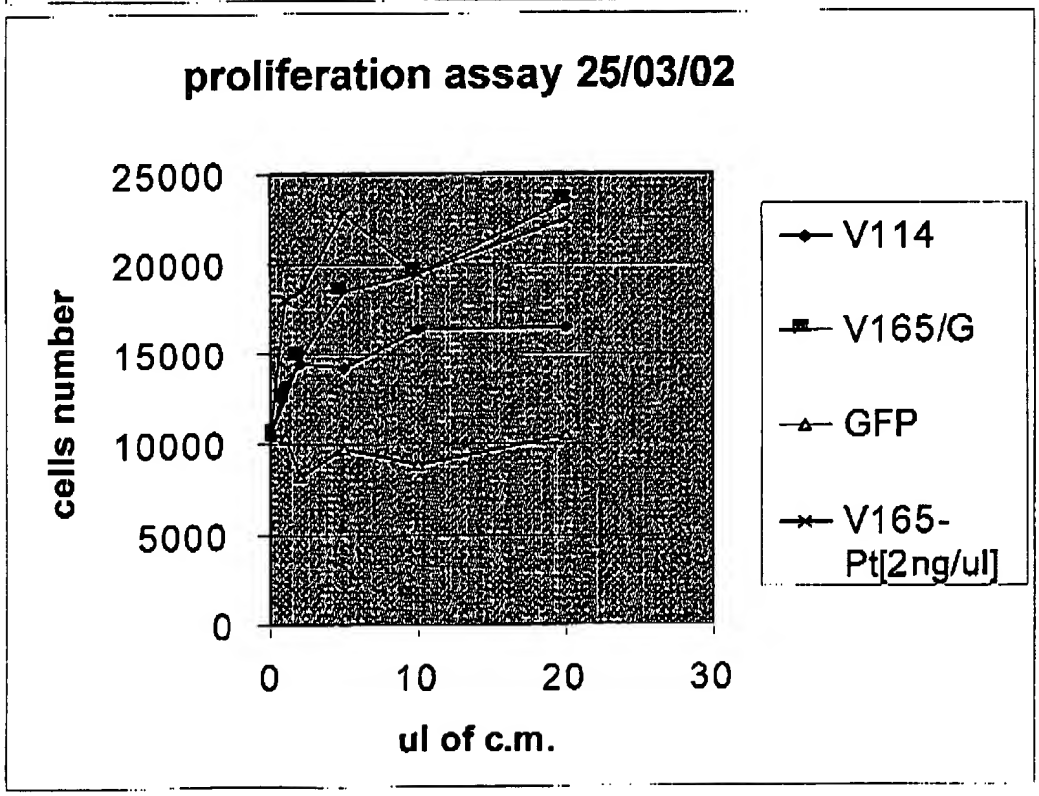
proliferation assay 25/03/02
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20000
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0 10 20 30
ul of c.m.
V114
V165/G
GFP
V165-Pt[2ng/ul]

Figure 5: Proliferation assay with conditioned medium of rAd *$VEGF_{114}$* infected EC. EC were seeded at the concentration $2x10^4$ cells/ well in 24 wells plate pre-coated with fibronectin in M199 containing 5% FCS. Increasing volumes of conditioned medium from adenovectors infected EC containing $VEGF_{114}$, VEGF or control were added each other day. Proliferation rate was detected 6-7 days after infection by cell counting using cell coulter.

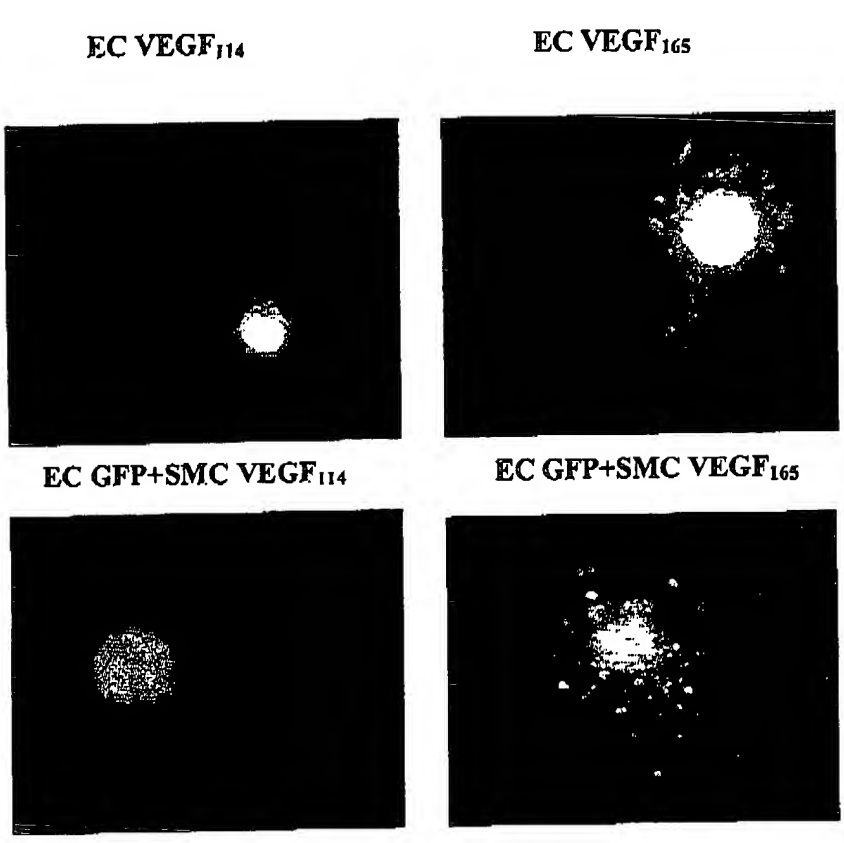

Figure 6: In-vitro angiogenesis assay.– sprouting assay in collagen

A. 3-Dimentional *in-vitro* angiogenesis with collagen gel-embedded spheroids of retrovirus $VEGF_{165}$/GFP or $VEGF_{114}$/GFP transduced endothelial cells. The spheroids were generated and embedded into collagen gels as described in materials and methods. The spheroids were incubated in collagen gels at 37°C, 5% CO_2 for 48 hr and documented by digital video camera (DXM1200 Nikon, Japan).

B. 3-Dimentional *in-vitro* angiogenesis with collagen gel-embedded coculture spheroids of retrovirus GFP transduced EC and $VEGF_{165}$/GFP or $VEGF_{114}$/GFP retro transduced SMC. EC were tagged

with DiI-291 red fluorescent marker prior to mixing. Coculture spheroids were generated and embedded into collagen gels as described in materials and methods. The spheroids were incubated in collagen gels at 37°C, 5% CO_2 for 48 hr and documented by digital video camera (DXM1200 Nikon, Japan).

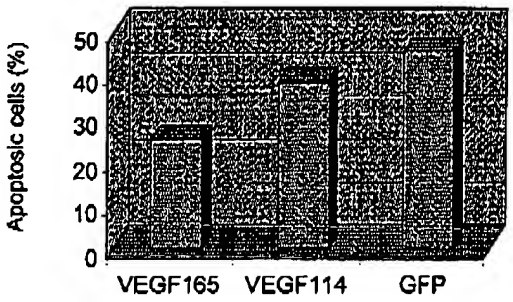

Figure 7: Apoptosis rate in recombinant retro transduced EC
EC were tranduced with recombinant retro $VEGF_{114}$–GFP, $VEGF_{165}$–GFP or GFP viruses. The assay was performed as described in methods. FACScan flow cytometer analysis was preformed to detect Annexin V-PE staining.

Conclusions

From the described study it can be concluded that expression of $VEGF_{114}$ have definitely effect on EC proliferation and apoptosis, but it is less potent than $VEGF_{165}$. In addition to the proliferation and apoptosis experiment, 3-dimentional *in-vitro* angiogenesis assay was preformed, in which $VEGF_{114}$ had no effect on EC sprouting. This observation supports the hypothesis about less potency of $VEGF_{114}$ comparatively to $VEGF_{165}$.